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Combinatorial signalling controls *Neurogenin2* expression at the onset of spinal neurogenesis

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ABSTRACT

A central issue during embryonic development is to define how different signals cooperate in generating unique cell types. To address this issue, we focused on the function and the regulation of the proneural gene *Neurogenin2* (*Neurog2*) during early mouse spinal neurogenesis. We showed that *Neurog2* is first expressed in cells within the neural plate anterior to the node from the 5 somite-stage. The analysis of *Neurog2* mutants established a role for this gene in triggering neural differentiation during spinal cord elongation. We identified a 798 base pair enhancer element (*Neurog2-798*) upstream of the *Neurog2* coding sequence that directs the early caudal expression of *Neurog2*. Embryo culture experiments showed that Retinoic Acid (RA), Sonic hedgehog (Shh) and Fibroblast Growth Factor signals act in concert on this enhancer to control the spatial and temporal induction of *Neurog2*. We further demonstrated by transgenesis that two RA response elements and a Gli binding site within the *Neurog2-798* element are absolutely required for its activity, strongly suggesting that the regulation of *Neurog2* early expression by RA and Shh signals is direct. Our data thus support a model where signal integration at the level of a single enhancer constitutes a key mechanism to control the onset of neurogenesis.

Introduction

During chick and mouse development, epiblast cells lying adjacent to the anterior primitive streak give rise to spinal cord progenitors (Brown and Storey, 2000; Cambray and Wilson, 2002, 2007; Mathis et al., 2001; Mathis and Nicolas, 2000). This region can be considered as a stem zone containing self-renewing progenitors which contribute to the formation of the spinal cord as the node regresses (Mathis and Nicolas, 2000). Fibroblast Growth Factor (FGF) signalling maintains the integrity and the undifferentiated state of progenitors within this region (Ciruna and Rossant, 2001; Mathis et al., 2001). From embryonic day 8 in the mouse (E8), some progenitors escape the influence of high FGF signalling (Mathis et al., 2001), reside transiently in the neural plate anterior to the node (also called pre-neural tube (pNT); Diez del Corral et al., 2002) and then populate the newly formed spinal cord. In this location the process of neurogenesis is initiated. Spinal progenitors then acquire (i) markers of the dorso-ventral (DV) axis including homeodomain (HD) and basic helix–loop–helix (bHLH) containing genes (for example, *Pax6* and *Olig2*, respectively) (ii) generic neuronal features mediated by a cascade of proneural bHLH transcription factors (Bertrand et al., 2002; Guillemot, 2007; Lupo et al., 2006). In parallel, progenitors identity along the antero-posterior (AP) axis, assigned in part by the combination of *Hox* genes they expressed, is stabilized and refined (e.g. Stern et al., 2006).

The initiation of these events is temporally and spatially regulated by multiple signalling pathways emanating from the node, the paraxial and axial mesoderm (Diez del Corral and Storey, 2004). Hence, convergent activities of FGFs, Glial cell line-derived neurotrophic factor (Gdnf11) and Retinoic Acid (RA) signals establish and refine the Hox-c-dependent positional identity of motor neurons (Liu et al., 2001). In parallel, RA and Sonic hedgehog (Shh) signalling pathways promote the emergence of a ventral expression pattern of homeodomain containing genes while FGF signalling represses these genes (Bertrand et al., 2000; Diez del Corral et al., 2002; 2003; Molotkova et al., 2005; Novitch et al., 2003; Wilson et al., 2004). Some evidence suggests that the initial steps of neurogenesis rely on multiple cross-interactions between these signalling pathways. However, the molecular mechanisms underlying such cross-talk remain an open question. For instance, the specification of motor neuron progenitors in response to Shh requires an active state of retinoic signalling (Novitch et al., 2003). Other data suggest that the effects of

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FGF signalling on spinal specification and differentiation could be mediated in part by a repression of the expression of some of the components of the RA and Shh signalling pathways. Indeed, FGFs induce a downregulation of the expression of *Retinaldehyde dehydrogenase 2* (*Raldh2*) encoding for an enzyme responsible for all RA synthesis in the early mouse embryo, *Retinoic acid receptor β* (*Rarβ*), *Shh* itself and *Patched2* (*Ptch2*) encoding for one of the Shh receptors (Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007).

To better understand how signalling pathways converge to trigger early steps of spinal cord development, we focused our study on the function and regulation of the proneural gene *Neurogenin2* (*Neurog2*). This gene plays essential roles during spinal neurogenesis. First, it is involved in controlling the timing of neural differentiation (Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001). This activity depends on the ability of Neurog2 to suppress the expression of *Sox2* and *Sox3* genes, possibly by inducing the vertebrate ortholog of the fly *gcm* (Bergsland et al., 2006; Bylund et al., 2003; Sandberg et al., 2005; Soustelle et al., 2007). In addition Neurog2 activates *SoxC* genes which in turn regulate the expression of pan-neuronal proteins (Bergsland et al., 2006). Second, Neurog2 influences the generation of specific neural subtypes. For example, in the motor neuron progenitor domain Neurog2, together with Olig2, promotes motor neuron differentiation. According to the current model, the choice between differentiating into a motor neuron or remaining a progenitor depends on the ratio of Olig2 repressor to Neurog2 activator (Lee et al., 2005; Lu et al., 2002; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Zhou and Anderson, 2002). Furthermore, the role of Neurog2 in specifying motor neurons relies in part on GSK3 mediated phosphorylations of the protein which allow the binding of Neurog2 to LIM homeodomain transcription factors. Notably, this post-translational modification is not required for its ability to induce neural differentiation (Ma et al., 2008).

Although Neurog2 function has been widely analysed, the time of its onset and its early function remained unknown. In this study, we show that *Neurog2* is first expressed in the E8.5 mouse embryo, within the neural plate anterior to the node where it controls the onset of spinal neurogenesis. We demonstrate that this early expression is driven by a single enhancer and that its activity is modulated in a combinatorial manner by RA, Shh and FGF signals. Furthermore we show that RA and Shh signalling pathways regulate *Neurog2* early expression via two RA response elements and one Gli binding site.

Materials and methods

Mouse lines and generation of transgenic mice

Mice heterozygous for the *Neurog2*^{KIlacZ} allele (Fode et al., 2000) were intercrossed to generate homozygous mutant embryos. *Neurog2-E2:: lacZ* transgenic mice have been described (Scardigli et al., 2001). Mice carrying the *Shh* mutation and the *Rare-lacZ* transgene were kindly provided R. Zeller and J. Rossant respectively. Transgenic mice were generated by standard procedures using fertilized eggs from FVBN embryos and founder animals were genotyped as described (Scardigli et al., 2001).

Neurog2-E2.1, *Neurog2-E2.2*, *Neurog2-E2.3*, *Neurog2-E2.4* and *Neurog2-798* sequences were amplified by PCR, a NotI and a SpeI restriction sites (underlined, see below) were added at the end of the 5′ and 3′ oligonucleotides respectively, to allow cloning of the resulting PCR fragment into the β*globin-lacZ* plasmid (Yee and Rigby, 1993). Numbers give the coordinate of the first base pair of the oligonucleotide based on the NCBI *Mus musculus* genome build 37.1 on chromosome 3.

E2.1 (fw) ATAAGAATGCGGCCGCCGGGAGAGCCAGCTCC (127, 329, 157)
(rev) GGACTAGTCCACTGCCTCAAGCCTCGGAGTGTGC (127, 330, 867)

E2.2 (fw) ATAAGAATGCGGCCGCACTTACCAGACCTGG (127, 330, 795)
(rev) GGACTAGTCTCGTCTCTGTCTAATCCTCTC (127, 332, 571)

E2.3 (fw) ATAAGAATGCGGCCGCAAGTTCCAGACCAGTC (127, 332, 571)
(rev) GGACTAGTGTCATCTGGGACCGGTCGCGGC (127, 334, 660)

E2.4 (fw) ATAAGAATGCGGCCGCGACCGGTCCCAG (127, 334, 636)
(rev) GGACTAGTCACTGCGTCTAGAGCGATGG (127, 336, 446)

798 (fw) ATAAGAATGCGGCCGCTAACTTAACTTTCTCGCC (12, 331, 316)
(rev) GGACTAGTTTCACTGAGAAAACGAAAAGGC (127, 332, 114)

In order to replace the β*globin* promoter by the endogenous *Neurog2* promoter, two complementary oligonucleotides harbouring SpeI and NcoI restriction (underlined, see below) at the 5′ and 3′ end respectively and corresponding to the conserved sequence (72 bp) around the TATA box between the human, chimpanzee, rat and mouse *Neurog2* regulatory sequence were hybridised and cloned into the *Neurog2-798* β*globin-lacZ* plasmid.

(fw) CTAGTGAGGAGGGGGCCCCGGGGCAGATCTGATTGTTTTCTTGGTGGTATATAA
GGGGTTTTAAGGAGAGTCGTGTGC
(rev) CATGGCTCACGACTCTCCTTAAAACCCCTTATATACCACCAAGAAAACAATCAGA
TCTGCCCCGGGGCCCCCTCCTCA

The design of null point mutations in the putative the *Rares*, the *Gli* and *Cdx* binding sites (BS) have been made according to Charite et al., 1998; Gustafsson et al., 2002; Marshall et al., 1994; the mutated bases appear in red:

Rare DR2 (fw) GCCCGCTTCGACATTTGTACTTCTTAAAGTAGGATTGC
(rev) ATCCTACTTTAAGAAGTACAAATGTCGAAGCGGGC
Rare DR5 (fw) TTTAGACAAGTACCGCTAATGTCGGGCGCGG
(rev) GCGCCCGACATTAGCGGTACTTGTCTAAAGG
Gli BS (fw) GCAAACTTTCAGACTGCAGAAGTGCCTCTTG
(rev) CAAGAGGCACTTCTGCAGTCTGAAAGTTTGC
Cdx BS (fw) GTGCCTCTTGAATTTACCCAAGTTAAAAGAGTGC
(rev) CTTTTAACTTGGGTAAATTCAAGAGGCACTTGTGTGG

The generation of point mutations was achieved by two rounds of PCR using internal oligonucleotides containing the appropriate mutations and external oligonucleotides used to amplify the *Neurog2-798* element. The mutation of the two *Rares* has been done sequentially. The sequences of the PCR amplification products were checked by sequencing.

Embryos culture experiments

Pregnant females were sacrificed at E8 and dissected embryos with intact yolk sacs were cultured in culture medium (rat serum, tyrode solution; 1:1) in 5 ml tubes (1 ml medium/tube; 8–10 embryos/tube). Recombinant mouse Shh-N (R and D systems) was rehydrated in culture medium at 2.5 μM. SU5402 (Calbiochem) and BMS493 (Bristol-Myers-Squibb) were dissolved in DMSO at 85 mM and 5 mM, respectively. Cyclopamine (LC laboratories) was rehydrated in a 45% solution of hydroxypropyl-beta-cyclodestrin (HBC, Sigma) in phosphate buffer at 2.42 mM. All-*trans*-RA (AT-RA; Biomol) was dissolved in 95% EtOH at 20 mM. For each drug, control embryos were cultured in presence of the drug's vehicle at the appropriate concentration. The efficacy of each drug was tested by analyzing the expression of target genes for each signalling pathways (Suppl. Fig. 2). Cultures were performed in a water-saturated roller-tube incubator at 37 °C, 5% CO_2 and 20% O_2. After culture, the embryos were fixed overnight at 4 °C in 4% paraformaldehyde and processed for *in situ* hybridization analysis. Gene expression patterns were always compared between embryos processed in the same culture experiment.

In situ hybridization, X-gal assays and immunohistochemistry

Whole-mount *in situ* hybridizations were performed using an Intavis InSitu Pro robot (*http://www.eumorphia.org/EMPReSS/servlet/EMPReSS.Frameset*). Gene expression patterns were always compared between embryos processed in the same robotic run. The probes used for *in situ* hybridisation were the following: *Neurog2* (Gradwohl et al., 1996); *lacZ* (Bonnerot et al., 1987); *Dll1* (Bettenhausen et al., 1995); *Hes5* (Akazawa et al., 1992); *Olig2* (Zhou and Anderson, 2002).

For immunochemistry, early somite-stage embryos were fixed in 4% paraformaldehyde for 30 min at 4 °C and were processed for immunohistochemistry as described (Scardigli et al., 2003). Primary antibodies incubations were done in a solution of 10% foetal calf serum, 3% BSA in phosphate buffer. WM neurofilament immunohistochemistry was performed as described (Vermot et al., 2005). The following antibodies were used: mouse monoclonal anti-Foxa2 (Developmental Studies Hybridoma Bank) and rabbit anti-mNeurog2 (kindly provided by Prof. Masato Nakafuku, Cincinnati Children's Hospital; Mizuguchi et al., 2001). Alexa 488- and Alexa 594-coupled secondary antibodies were purchased from Molecular Probes. Whole mount embryos were flat mounted between slide and coverslip in Aquapolymount (Polysciences Inc.) and analysed using a Leica M420 macroscope. The sectioned embryos were analyzed using a Leica M420 macroscope or a Leica Sp2MP confocal microscope.

Electrophoretic mobility shift assays

EMSA were performed essentially as described (Germain et al., 2002). For supershift assays, 1 µl of each antibody (9E10 and anti HA) was used as described (Gustafsson et al., 2002). The following oligonucleotides were used:

DR2-ctl	(fw) TCGAGGGTAGGGGTCACAGGGTCACTCG
	(rev) CGAGAGACCCTGTGACCCCTACCCTCGA
DR2-*Neurog2*	(fw) GCTTTGAGCTTTTGTTCTCTTAAAGTAG
	(rev) CTACTTTAAGAGAACAAAAGCTCAAAGC
DR2-*Neurog2* mt	(fw) GCTTCGACATTTGTACTTCTTAAAGTAG
	(rev) CTACTTTAAGAAGTACAAATGTCGAAGC
DR5-ctl	(fw) TCGAGGGTAGGGGTCACCGAAAGGTCACTCG
	(rev) CGAGTGACCTTTCGGTGACCCCTACCCTCGA
DR5-*Neurog2*	(fw) CCCTTTAGACAGTTCACGCTATGGACAGGCG
	(rev) CGCCTGTCCATAGCGTGAACTGTCTAAAGGG
DR5-*Neurog2* mt	(fw) CCCTTTAGACAAGTACCGCTAATGTCGGGCG
	(rev) CGCCCGACATTAGCGGTACTTGTCTAAAGGG
Gli-ctl (myf5)	(fw) GGGAAAAAACGACCACCAAGAAACACAGT
	(rev) ACTGTGTTTCTTGGTGGTCGTTTTTTCCC
Gli-*Neurog2*	(fw) CAAACTTTCAGACCACACAAGTGCCTCTT
	(rev) AAGAGGCACTTGTGTGGTCTGAAAGTTTG
Gli-*Neurog2* mt	(fw) CAAACTTTCAGACTGCAGAAGTGCCTCTT
	(rev) AAGAGGCACTTCTGCAGTCTGAAAGTTTG

Results

Neurog2 regulates the onset of spinal neurogenesis

A detailed examination of *Neurog2* expression by RNA *in situ* hybridization at the onset of neurogenesis in the spinal cord revealed that this gene is first expressed in scattered cells of the neural plate anterior to the node (referred further as pre-neural tube, pNT) from the 5 somite-stage (5ss) onwards. We confirmed the presence of Neurog2 protein among pNT cells by immunofluorescence (Fig. 1D). In this region, the progenitors are restricted to a neural fate characterized by the expression of the pan-neuronal marker *Sox2* and the absence of mesodermal markers such as the *T* gene (Suppl. Fig. 1 and data not shown, see also Delfino-Machin et al., 2005). However, they do not express the ventral specification markers such Pax6, *Nkx2.2* and *Olig2* (Suppl. Fig. 1 and data not shown). Based on lineage analysis, cells within the pNT contribute to the formation of the spinal cord during the elongation of trunk structures (Cambray and Wilson, 2002, 2007; Mathis and Nicolas, 2000). Therefore, induction of *Neurog2* may represent an early step of commitment of the self-renewing spinal progenitors. From the 5ss onwards expression of *Neurog2* is also apparent in the prospective diencephalon (Figs. 1A, A′). It then extends to the walls of the neural tube at around 7ss, and to the hindbrain by 9ss (Figs. 1B, C).

To determine the early function of Neurog2, we examined the trunk region of *Neurog2* mutant embryos (*Neurog2*$^{KILacZ/KIlacZ}$, further called *Neurog2*$^{-/-}$). We first analysed the consequence of loss of *Neurog2* on Delta-Notch signalling (Louvi and Artavanis-Tsakonas, 2006). *Dll1* expression in the caudal neuroepithelium parallels *Neurog2* expression, with scattered *Dll1* positive cells appearing at 5ss in the pNT and with a delay in the neural tube (Figs. 1E, F and data not shown). In *Neurog2*$^{-/-}$ embryos, *Dll1* expression is missing from the pNT and the spinal cord until E9.0, whereas it is still observed in the forming brain (Figs. 1G, G′). At E9.5, *Dll1* remains absent from the caudal-most part of *Neurog2* mutants (Fig. 1H′), but *Dll1* starts to be expressed in the spinal cord at brachial level (Fig. 1H). Expression of *Hes5* a Notch target and effector, is absent from the pNT and is severely reduced in the spinal cord of *Neurog2* mutants, compared to wild type (WT) embryos, at all stages analyzed (Figs. 1I–L′). In addition, the number of differentiated neurons monitored by the expression of neurofilament is decreased in *Neurog2*$^{-/-}$ embryos even at brachial level (Figs. 1M–N′). Notably the size of the ventral horn where the first post mitotic motor neurons migrate is severely reduced in mutants at that stage (dashed line, Figs. 1M′–N′; see also Fig. 2, Ma et al., 2008 for a later stage). The number of differentiated neurons in the ventral part of the neural tube of *Neurog2* mutants eventually reaches the levels of those in WT embryos at E10.75 (Scardigli et al., 2001). This recovery is likely to be due to the appearance in the neural tube of *Neurog1* at E9.5 both in WT and *Neurog2*$^{-/-}$ embryos (data not shown). Therefore, *Neurog2* is likely to be the only proneural gene in the caudal region of mouse embryos and in contrast to later stages (Scardigli et al., 2001), its function cannot be compensated by Neurog1 at the onset of neurogenesis. Interestingly, in chick the proneural genes involved in the initial steps of spinal neurogenesis differ. Indeed, both *Neurog1* and *Neurog2* are excluded from the medial pNT of chick embryos and therefore might have a limited role in initiating the lateral inhibition process (Akai et al., 2005). Conversely, *Cash4*, a chicken-specific proneural gene is expressed within the caudal most part of this tissue and has the capacity to induce *Dll1* expression (Akai et al., 2005; Henrique et al., 1997). Importantly, expression of *Olig2*, *Pax6* and *Nkx2.2* is similar in early somite-stage WT and *Neurog2*$^{-/-}$ embryos (data not shown) suggesting that Neurog2 is not required for induction of these factors. Together these results show that in the mouse from the 5ss onwards Neurog2 function in the pNT and the newly formed spinal cord is necessary for the commitment and the differentiation of spinal progenitors. In order to gain new insight into the onset of neurogenesis we focused our study on the mechanisms that induce early expression of *Neurog2*.

Multiple signals determine the onset of Neurog2 expression

We assayed for instructive factors, which act independently of the Notch-mediated lateral inhibition process that could be involved in the induction of *Neurog2* expression (Bertrand et al. 2002). In spite of their role in the regulation of *Neurog2* expression in the motor neuron progenitor domain (Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2003, 2001), Pax6 and Olig2 are unlikely to be required for *Neurog2* early expression, as indicated by their expression profile which is excluded from the domain of *Neurog2* induction (Suppl. Fig. 1). Moreover *Neurog2* expression is maintained in *Pax6* mutant embryos (Suppl. Fig. 1; Bel-Vialar et al., 2007). We

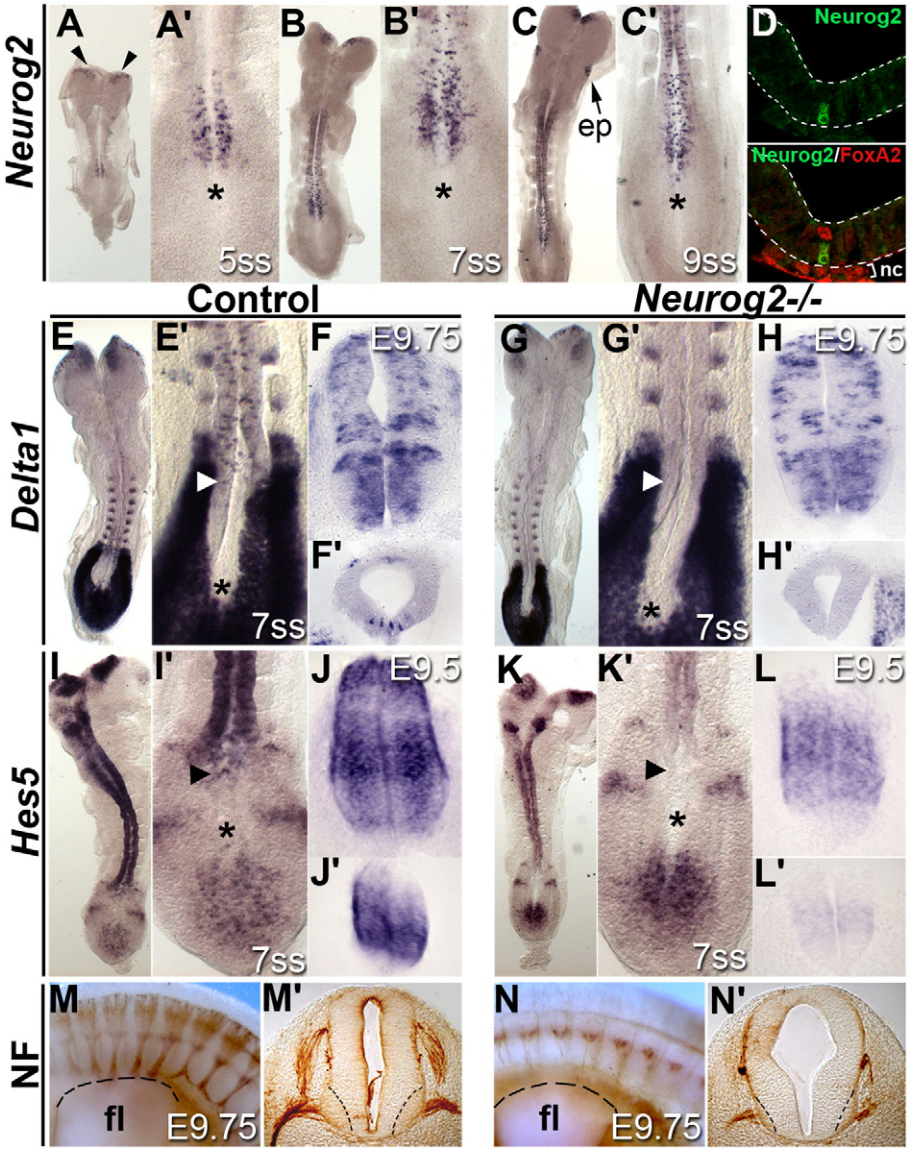

Fig. 1. Neurog2 triggers spinal neurogenesis at early somite-stages. (A–C) *In situ* hybridizations (ISH) on whole mount (WM) embryos showing *Neurog2* early expression. A′, B′, C′: higher magnifications of embryos in panels A–C. (D) Neurog2+ cells detected on transverse sections at the level of the pNT by immunochemistry are intermixed with FoxA2+ cells. The dashed line underlines the neuroepithelium. (E–L′) In *Neurog2*$^{-/-}$ mice expression of *Delta1* and *Hes5* monitored by ISH is absent from the pNT and appears with a delay in the anterior spinal cord; WM embryos (E, G, I, and K) and transverse sections at brachial (F, H, J, and L) and lumbar (F′, H′, J′, and L′) levels. The arrow heads (E′, G′, I′, and K′) points to the pNT where *Neurog2* expression is initiated. (M, N′) A reduction in the number of differentiated neurons expressing neurofilament (NF) is seen *in Neurog2*$^{-/-}$ spinal cord; WM embryos (M, N) and transverse sections at brachial level (M′, N′), the dashed lines underlines the ventral horn. (nc) notochord; (asterisk) node; (fl) forelimb; (fine arrow head) diencephalon; (ep) epibranchial placode; (thick arrow head) pNT.

then asked whether *Neurog2* induction requires extrinsic signals from the neural plate by culturing early somite-stage caudal neuroepithelium from *Neurog2*$^{KIlacZ/+}$ embryos on collagen gel. The isolation of caudal neuroepithelium from the surrounding tissues at the 4–7ss prevented *Neurog2* induction. In contrast, culturing isolated neural tube after the onset of *Neurog2* expression (10–12ss) had no effect on its expression (Suppl. Fig. 1). Therefore, *Neurog2* induction, but not its maintenance, requires extrinsic factors not present in the neuroepithelium. This result is in agreement with the requirement of extrinsic factors for *NeuroM* expression in chick spinal cord (Diez del Corral et al., 2002). This gene encodes a chicken specific bHLH protein expressed exclusively in post-mitotic neurons (Roztocil et al., 1997). The initiation of the whole generic neurogenesis program both in mouse and chick seems thus to be under the control of cell-interactions between the neuroepithelium and the flanking mesoderm.

Among the extracellular signals that regulate spinal development, we chose to analyze the function of the RA, FGF and Shh signals in the induction of *Neurog2* expression, as the three signalling pathways were active in the pNT (Figs. 2A–D). Indeed, pNT cells were expressing the RA-responsive transgene, *Rare-lacZ* (Rossant et al., 1991), as well as *Patched* 1 (*Ptch1*) and low levels of *Sprouty2* (*Spry2*), two downstream targets of Shh and FGF signalling, respectively (Goodrich et al., 1999; Minowada et al., 1999; see also Suppl. Fig. 2). We first assessed the putative contribution of these signals in the regulation of *Neurog2* expression by culturing early somite-stage embryos (i.e. before the onset of *Neurog2* expression) for 6 h in media containing either agonists or antagonists of these signalling pathways. This short time point was chosen in order to identify direct regulations of *Neurog2* early expression and to minimize the indirect effects that could be induced by modulating developmentally important signals both on the neuroepithelium itself or/and the adjacent tissues.

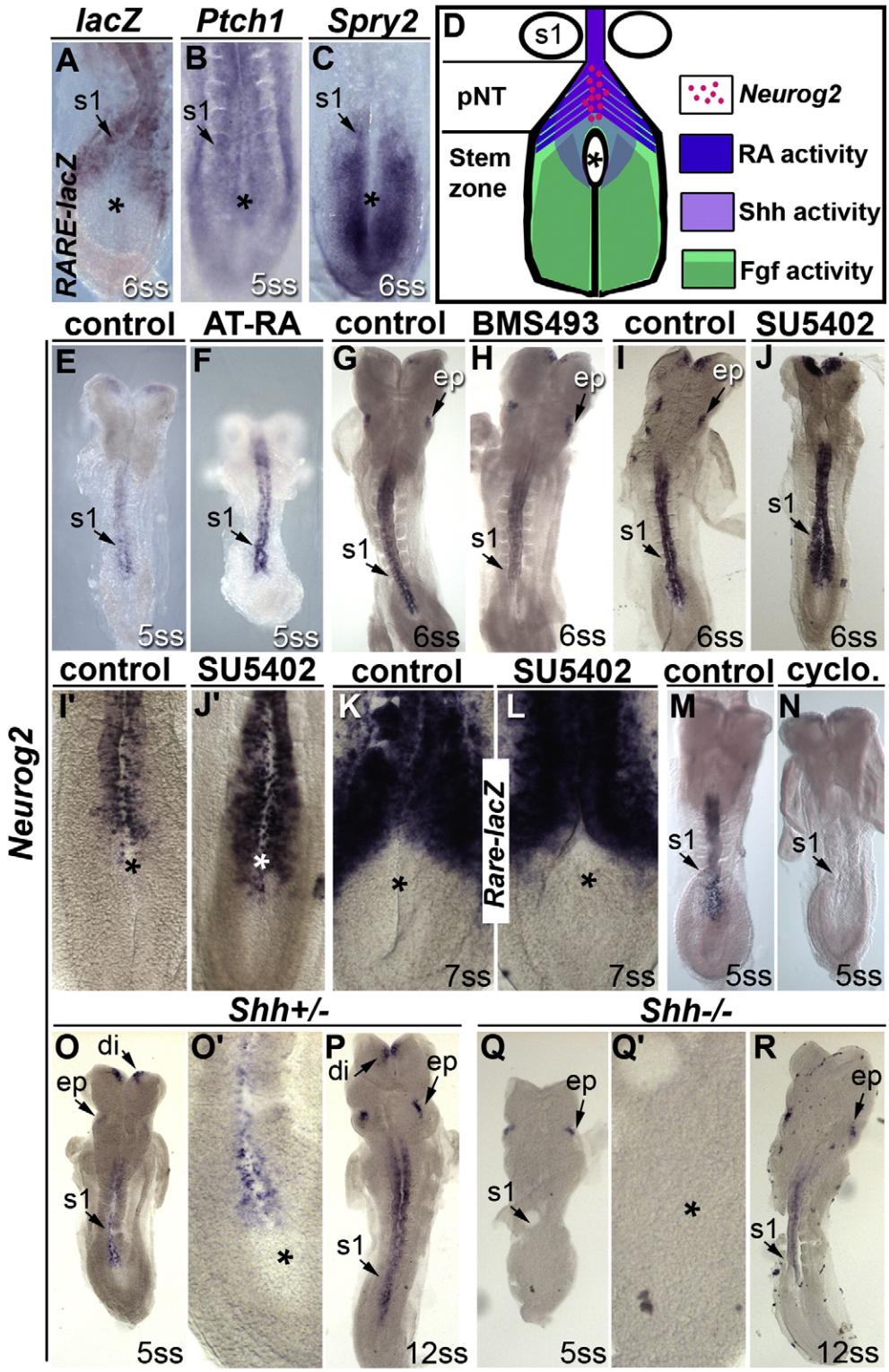

Fig. 2. RA, FGF and Shh signalling modulates *Neurog2 early* expression. (A–C) WM ISH for *lacZ* (A), *Ptch1* (B) and *Spry2* (C) mRNA in 5–6ss mouse embryos carrying the *Rare-lacZ* transgene (A) or WT (B, C) showing respectively the presence of active RA, Shh and FGF signalling within the pNT and the forming neural tube. (D) Schematic representation of RA, Shh and FGF activities within the caudal plate of mouse embryos. In the medial part of the pNT where *Neurog2* is induced, both RA and Shh activities are initiated, whereas Fgf signalling is fading. (E–N) WM ISH of mouse embryos collected at the 2–5ss and cultured for 6 h in the presence of drugs and hybridized with *Neurog2* (E–J′; M, N) and *lacZ* RNA (K, L) probes. (E, F) 2×10^{-7} M AT-RA induces ectopic expression of *Neurog2* in the pNT and neural tube. (G, H) Conversely, blocking RA using 5×10^{-6} M BMS493 decreases *Neurog2* expression. (I–L) Reducing FGF signalling using 5×10^{-5} M SU5402 increases the number of *Neurog2*+ cells in the pNT (I, J′) without expanding RA activity monitored by the *Rare-lacZ* transgene (*lacZ* RNA probe) (K, L). (M, N) Reducing Shh signalling by the addition of 10^{-4} M cyclopamine reduces markedly *Neurog2* expression. (O–R) WM ISH on *Shh*$^{-/-}$ embryos reveals that *Neurog2* is absent from the pNT and remains very weak in the neural tube compare to WT embryos. (asterisk) node; (s1) last formed somite; (ep) epibranchial placode.

The addition of 2×10^{-7} M *all trans*-RA (AT-RA) to the culture medium led to an increase in the number of *Neurog2* expressing cells within the caudal neural tube (4 embryos out of 4; 4/4) compared to control embryos ($n=3$ Figs. 2E, F; see also Suppl. Fig. 2). Accordingly, inhibition of RA activity by 5×10^{-6} M BMS493, a pan-Rar antagonist (Germain et al., 2002), resulted in the absence of *Neurog2* expression in the pNT (9/10) and in a decrease of *Neurog2* expression in the neural tube (10/10). Expression in the head and epibranchial placodes (ep) was however unaffected (Figs. 2G, H; see also Suppl. Fig. 2). These results are in agreement with the finding that *Neurog2* expression is absent in the spinal cord of RA deficient embryos (Diez del Corral et al., 2003; Novitch et al., 2003; VR, ILR, PD in preparation). We then assessed the role of FGF signalling in regulating *Neurog2* expression by adding 5×10^{-5} M SU5402, an FGF receptor inhibitor to the culture medium (Mohammadi et al., 1997). *Neurog2* expression in the spinal cord was comparable in treated ($n=11$) and untreated ($n=16$) embryos (Figs. 2I–J). In contrast, its expression within the pNT was expanded both laterally and caudally towards the node region (10/11; Figs. 2I–J′). Of note, *Neurog2* was also absent from the epibranchial placodes (10/11; Figs. 2I–J). The ectopic expression of *Neurog2* in the pNT could be a consequence of an increase in RA activity, as in chick ectopic FGF signalling has been shown to repress the expression of some of the components of the RA signalling pathway (Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007; see introduction). This is, however, unlikely to be the case here since the pattern of RA activity using the RA-responsive transgene, *Rare-lacZ* was similar in controls ($n=6$) and embryos cultured in the presence of 5×10^{-5} M SU5402 ($n=5$) (Figs. 2K, L). These results thus suggest that FGF and RA signalling pathways regulate in an opposite manner *Neurog2* early expression, independently of each other.

To test the role of Shh on the onset of *Neurog2* expression, embryos were incubated with an active form of Shh protein (5×10^{-8} M Shh-N) or with 10^{-4} M cyclopamine, an inhibitor of Smoothened, a component of the Shh signalling pathway (Incardona et al., 1998). *Neurog2* expression was comparable in control and Shh-N treated embryos ($n=20$ and $n=11$, respectively; data not shown). In contrast, a fraction of embryos incubated with cyclopamine displayed a marked decrease or complete loss of *Neurog2* expression (5/14) compared to control embryos ($n=6$) (Figs. 2M, N). This suggests that the Hedgehog signalling pathway is required for early expression of *Neurog2*. We further confirmed this finding by analysing *Neurog2* expression in *Shh* mutants. *Neurog2* expression was completely absent from the pNT and the prospective diencephalon of 5–7ss *Shh* mutants (3/3) although it was present in the placode regions (Figs. 2O, O′, Q, and Q′). At 12ss, *Neurog2* expression remained absent from the pNT and the prospective diencephalon while a low level of *Neurog2* expression was detectable in the caudal neural tube (2/2; Figs. 2P, R). Thus, our results reveal a new role for Hedgehog signalling pathway in the initiation of spinal neurogenesis through the regulation of *Neurog2* expression.

Together, these findings indicate that RA, FGF and Shh signalling pathways, known to modulate spinal neurogenesis, provide positive and negative inputs into the regulation of one single gene in the pNT. Notably, *Neurog2* expression displays different requirements for the three signalling pathways in different parts of the nervous system. This observation suggests that early *Neurog2* expression results, as at later embryonic stages, from the modular activity of several enhancer elements (Scardigli et al., 2001; Simmons et al., 2001).

RA, FGF and Shh signals regulate Neurog2-E2 and Neurog2-798 enhancer activities

We next asked whether the signalling pathways modulating *Neurog2* early expression directly regulate *Neurog2* transcription. For this, we examined the activity at the onset of neurogenesis of several regulatory elements that drive *Neurog2* expression in different domains of the ventral spinal cord at E10.5 (Scardigli et al., 2001). We found that one of these elements, *Neurog2-E2*, drives expression of the *lacZ* reporter gene in a pattern identical to the early expression of *Neurog2* (Figs. 3A and 4A, B, D, and E). Other spinal *Neurog2* elements had no activity at this stage (data not shown). Similar to *Neurog2* expression, *Neurog2-E2* activity first appears in scattered cells of the anterior pNT at 5ss and this expression extends to the neural tube and the diencephalon as development proceeds (Figs. 4A, A′, B, B′, D, and E). In contrast to *Neurog2* expression, however, *Neurog2-E2* activity was not observed in the epibranchial placodes, the rhombencephalon and the midbrain at E8.5 and E9.5 (Figs. 4A, B, D, and E and data not shown). We then divided the E2 element into four fragments (*Neurog2*-E2.1- *Neurog2*-E2.4, see Materials and methods) and generated transient transgenic embryos with these regions. Only *Neurog2-E2.2:: lacZ* transgenic embryos ($n=3$) displayed a robust *lacZ* expression pattern similar to that of *Neurog2-E2* in the pNT and the developing spinal cord at E8.5 (data not shown). Phylogenic footprinting of the *Neurog2-E2.2* sequence using PromAn (Lardenois et al., 2006) identified a 798 base pair (bp) sequence in the *Neurog2-E2.2* element (*Neurog2-798*; Fig. 3A) located 4507 bp 5′ to the initiation codon that is highly conserved in the mouse, rat, dog, chimpanzee and human genomes (615 bp conserved out of 798 bp). We tested the activity of the *Neurog2-798* sequence by transgenesis and found that it was similar to *Neurog2-E2* enhancer activity in E8.5 and E9.5 transgenic embryos (Figs. 4C, C′, and F; Fig. 5B; data not shown).

We then investigated whether the activity of *Neurog2-E2* and *Neurog2-798* elements were modulated by RA, FGF and Shh signalling. For this, we cultured early somite-stage *Neurog2-E2:: lacZ* and *Neurog2-798:: lacZ* transgenic embryos in the presence of inhibitors of RA, FGF and Shh signalling pathways. Incubation of transgenic embryos with 5×10^{-6} M BMS493 led to the loss of reporter gene expression in the neural tube of both *Neurog2-E2:: lacZ* embryos (9/9) and *Neurog2-798:: lacZ* embryos (6/6) compared to controls ($n=11$ and $n=9$, respectively; Figs. 4G, H and Suppl. Fig. 2). This indicates that RA signalling is essential for the activity of the *Neurog2* regulatory elements as it is for *Neurog2* expression. Following incubation with 5×10^{-5} M SU5402, we observed a caudal expansion of *lacZ*-positive cells within the pNT in both *Neurog2-E2:: lacZ* embryos (3/3) and *Neurog2-798:: lacZ* embryos (4/5) compared to vehicle treated embryos ($n=3$ and $n=8$, respectively; Figs. 4I, J and Suppl. Fig. 2). This is similar to the effect of FGF inhibition on *Neurog2* expression and indicates that FGF signalling acts in the same manner on the *Neurog2* regulatory elements. Finally, the presence of 10^{-4} M cyclopamine in the culture medium led to a decrease in *lacZ* expression in *Neurog2-E2:: lacZ* embryos (8/9) and *Neurog2-798:: lacZ* embryos (7/8) compared to controls ($n=13$ and $n=12$, respectively; Figs. 4K, L and Suppl. Fig. 2). This demonstrates that Shh is required for the activity of the *Neurog2* elements. Cyclopamine treatments were much more efficient in disrupting the activity of the transgenes (*Neurog2-E2*: 88% *and Neurog2-798*: 87%) than that of *Neurog2* endogenous expression (35% and see above). One possible explanation for this discrepancy is the difference of the genomic context between these constructs. It is very likely that another enhancer(s) element(s) outside the *Neurog2-E2* element contributes independently of Shh signalling with the *Neurog2-798* element to the positive regulation of *Neurog2* early expression. Together these data show that *Neurog2-E2* and *Neurog2-798* elements are responsive to RA, FGF and Shh signals similar to the endogenous *Neurog2*. This strongly suggests that the expression of *Neurog2* in the forming neural tube is mediated by the *Neurog2-798* enhancer element.

RA and Shh signals converge at the level of Neurog2-798 enhancer

We next determined whether the *Neurog2-798* element was regulated directly by RA, FGF and Shh signalling pathways. For this, we

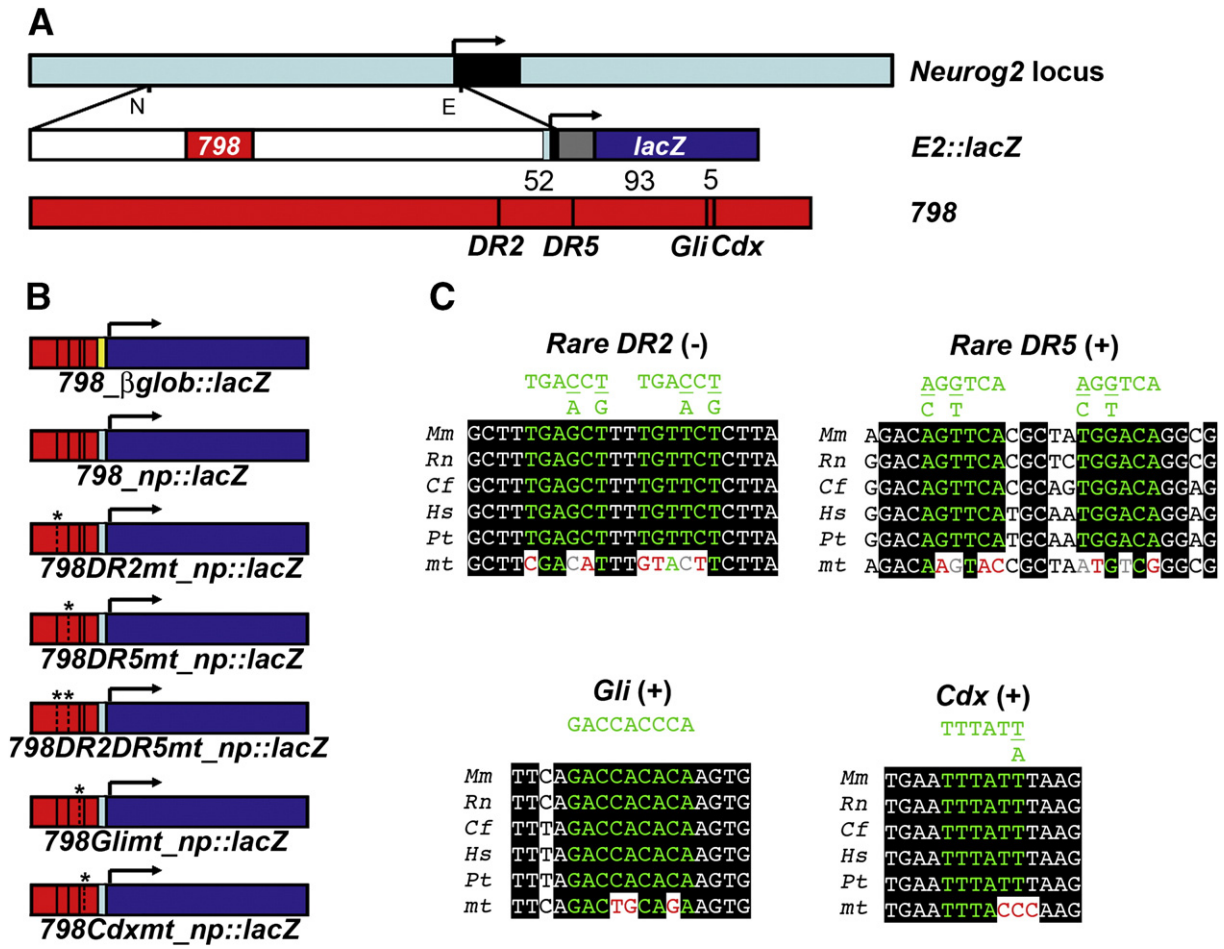

Fig. 3. Description of the *Neurog2-798* enhancer element. (A) Schematic representation of *Neurog2-E2:: lacZ* and *Neurog2-798:: lacZ* constructs. The lower bar indicates the relative position of the putative binding sites for Rar/Rxr heterodimers, Gli and Cdx transcription factors within the *Neurog2-798* element. The numbers corresponds to the number of bp between each binding sites. (B) Schematic representation of the different transgenic constructs described in the present work. All contained the endogenous *Neurog2* promoter (np) except *798_βglob:: lacZ* which contained the Human *βglobin* promoter. (C) Comparison of the sequences of the putative binding sites for Rar, Gli and Cdx transcription factors of the *Neurog2-798* element between Mus musculus (*Mm*), Rattus norvegicus (*Rn*), Canis familiaris (*Cf*), Homo sapiens (*Hs*) and Pan troglodytes (*Pt*). The upper sequences represent consensus binding sites, the lower line (mt) point mutations (red letters) generated at these sites and the black boxes the conservation between the sequences.

searched for conserved consensus binding sites for transcription factors acting downstream of these signalling pathways. Using the Matinspector program and Genomatix transcription factor weight matrices (http://www.genomatix.de), we identified in the *Neurog2-798* sequence two putative RA responsive elements (Rares) (type 2; DR2 and type 5; DR5), one putative *Gli* binding site and one putative *Cdx* site, which are conserved in the mouse, rat, dog, chimpanzee and human genomes (Figs. 3A, C). Interestingly, *Cdx* genes have been reported to mediate the activity of the FGF, Wnt and RA signalling pathways on *Hox* gene regulation in the posterior embryo (Lohnes, 2003).

We next examined the role of these putative binding sites on *Neurog2-798* activity. For this, we analysed the expression of reporter in transgenic mice harbouring *Neurog2-798:: lacZ* transgenes containing mutations in the binding sites that are expected to disrupt transcription factor binding (Figs. 3B, C). *lacZ* expression in transgenic embryos with a mutation in the *Cdx* binding site (*Neurog2-798Cdxmt:: lacZ*) was identical to that found in embryos with a WT transgene (Fig. 5A–C′). This shows that the *Cdx* binding site is not required for *Neurog2-798* enhancer activity. In contrast, mutation of the *Gli* binding site (*Neurog2-798Glimt*) resulted in absence of *lacZ* expression in the pNT (8/8) and the neural tube (7/8) of early somite-stage embryos (Fig. 5A). One of the *Neurog2-798Glimt:: lacZ* transgenic embryos showed weak *lacZ* expression in the neural tube (Fig. 5D, D′). This result thus shows that an intact *Gli* binding site is necessary for the full activity of *Neurog2-798* sequence. To determine whether Gli proteins can physically interact with this binding site we performed electromobility shift assays (EMSA). A myc tagged Gli protein (Stamataki et al., 2005) can indeed bind specifically *in vitro* to an oligonucleotide corresponding to the surrounding sequences of the *Gli* binding site of *Neurog2-798* (Fig. 5E, see legend for details).

We also explored the function of the two Rares by generating transgenic mice harbouring point mutations in the *Rare-DR2* (*Neurog2-798DR2mt:: lacZ*), the *Rare-DR5* (*Neurog2-798DR5mt:: lacZ*) or in both *Rares* (*Neurog2-798DR2DR5mt:: lacZ*). None of the transgenic mice harbouring mutation(s) in the *Rare* displayed any expression of the reporter gene (Fig. 5A and data not shown) showing that *Neurog2-798* enhancer activity requires the two *Rares*. We next tested by EMSA the ability of the *Rare-DR5* and *Rare-DR2* sequences to bind *in vitro* to Rar and retinoic X receptor (Rxr) heterodimers. We used receptors deleted in the activation domain (AB domain; RarΔAB/RxrΔAB) since the binding to *Rare* sequences is independent of this domain (Germain et al., 2002). We found that *Rare-DR5* oligonucleotides bind, albeit weakly, to RarbΔAB/RxrgΔAB heterodimers (Fig. 5G). In contrast, the *Rare-DR2* sequence was unable to bind significantly RarbΔAB/RxrgΔAB heterodimers (Fig. 5F, see legend for details). Together, the EMSA experiments and the transgenic analysis suggest that the two *Rares* may work in a cooperative manner, the binding of the *Rare-DR5* being a prerequisite

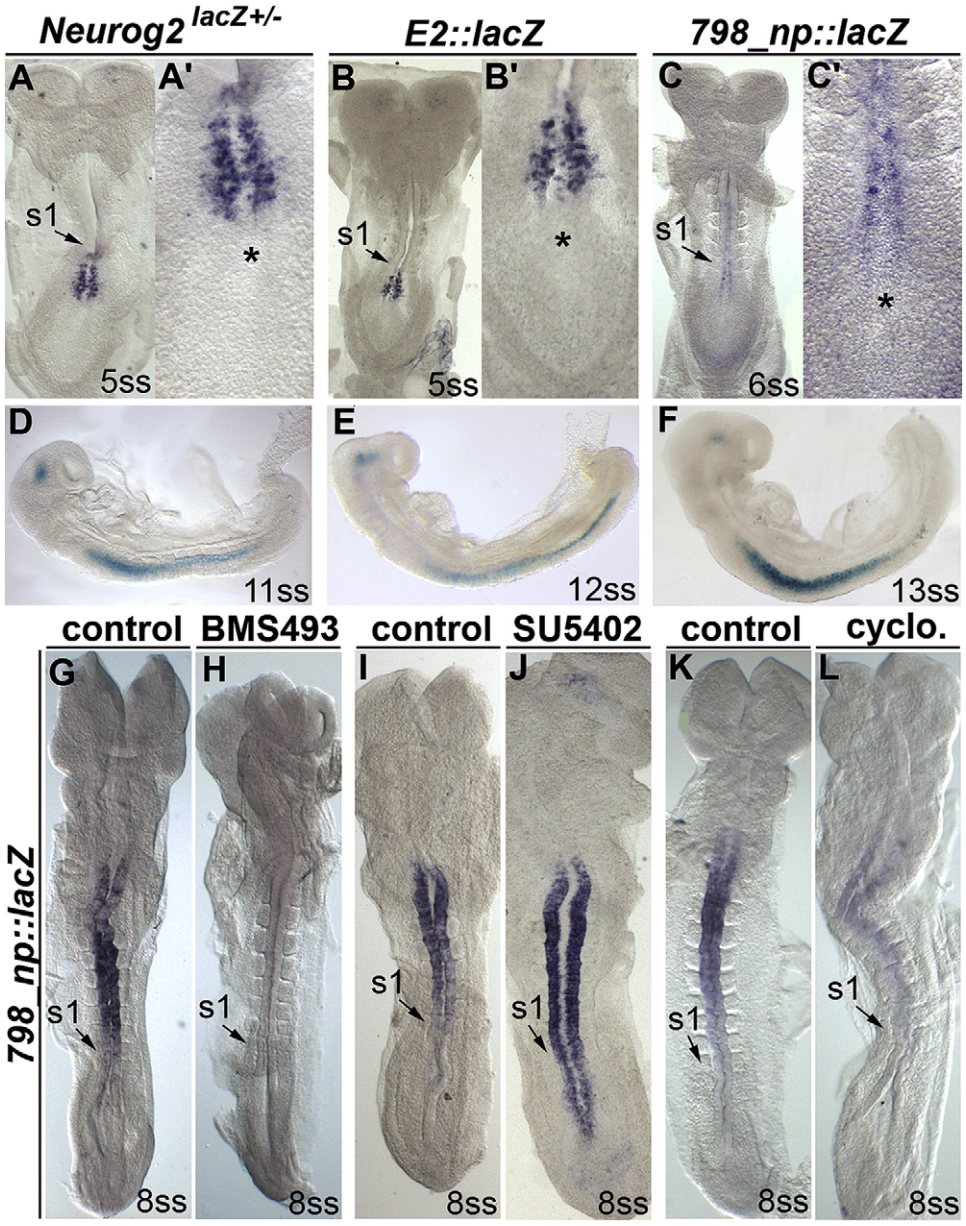

Fig. 4. RA, Shh and FGF signalling pathways modulate the activity of the *Neurog2-798* regulatory element. (A–F) The activity of *Neurog2-E2:: lacZ* and *Neurog2-798:: lacZ* in transgenic mice visualized using ISH for *lacZ* (B, C′) or WM X-gal staining (E, F) recapitulates *Neurog2* early expression seen in *Neurog2*$^{KIlacZ/+}$ embryos (A, A′, and D). (G–L) In embryo cultures (see Fig. 2), addition of 5×10^{-6} M BMS 493 or 10^{-4} M cyclopamine is sufficient to markedly reduce the activity of the *Neurog-798::lacZ* transgene (G, H, K, L), whereas reducing FGF signalling using 5×10^{-5} M SU5402 expands caudally the element activity (I, J). (asterisk) node; (s1) last formed somite.

for the binding of the *Rare*-DR2 (see also the discussion). However, we cannot exclude the possibility that factors others than Rar/Rxr heterodimers bind to the *Rare-DR2* binding site.

Discussion

The roles of multiple functional signalling pathways, including RA, Shh and FGF, in the control of the development of the spinal cord have been extensively studied. However, the mechanisms of integration of these signals at the level of neural precursors remained poorly defined. Conceptually, signalling pathways could directly interact to cross-regulate each other's pathway. Alternatively, these signals could be integrated at the level of the transcriptional regulation of individual genes. Our present study demonstrates that the later mode of signalling integration is responsible for the regulation of the expression of a key determinant of spinal neurogenesis, *Neurog2*. We propose that this mechanism can be responsible in part for the precise temporal and spatial organization of neuronal subtype identities within the vertebrate spinal cord.

Our data show that the *Neurog2-798* enhancer element is responsible for the early expression of *Neurog2* and that the activity of this element is modulated by RA, Shh and FGF signalling pathways. First, our findings provide several lines of evidence for a strict requirement of RA and Shh signals for the enhancer element activity. Activated by these signals, the downstream Rar/Rxr heterodimers and Gli transcription factors act most probably through the identified *Rares* and *Gli* binding site and trigger *Neurog2* expression. Second, the analysis of embryos cultured for a short period of time in the presence of an FGF receptor inhibitor suggests that FGF signals play a role in the restriction of the *Neurog2* expression domain via modifying the activity of the *Neurog2-798* enhancer element. Intriguingly, no conserved binding sites for known downstream components of the FGF signalling pathway have been identified within the enhancer element, raising the issue of the identity of the transcriptional

A

Transgenic lines	insertions T.S	E8.5	E10.5	E12.5
798_βglob::lacZ	0.1	+	++	++
798_np::lacZ	0.1	+	++	-
798Cdxmt_np::lacZ	0.1	+	++	-
798Glimt_np::lacZ	7.1	+/- (1/8) - (7/8)	+/- (1/1)	nd
798DR2mt_np::lacZ	2.1	- (3/3)	-(1/1)	-(1/1)
798DR5mt_np::lacZ	2.1	- (3/3)	-(1/1)	-(1/1)
798DR2DR5mt_np::lacZ	12.0	- (12/12)	nd	nd

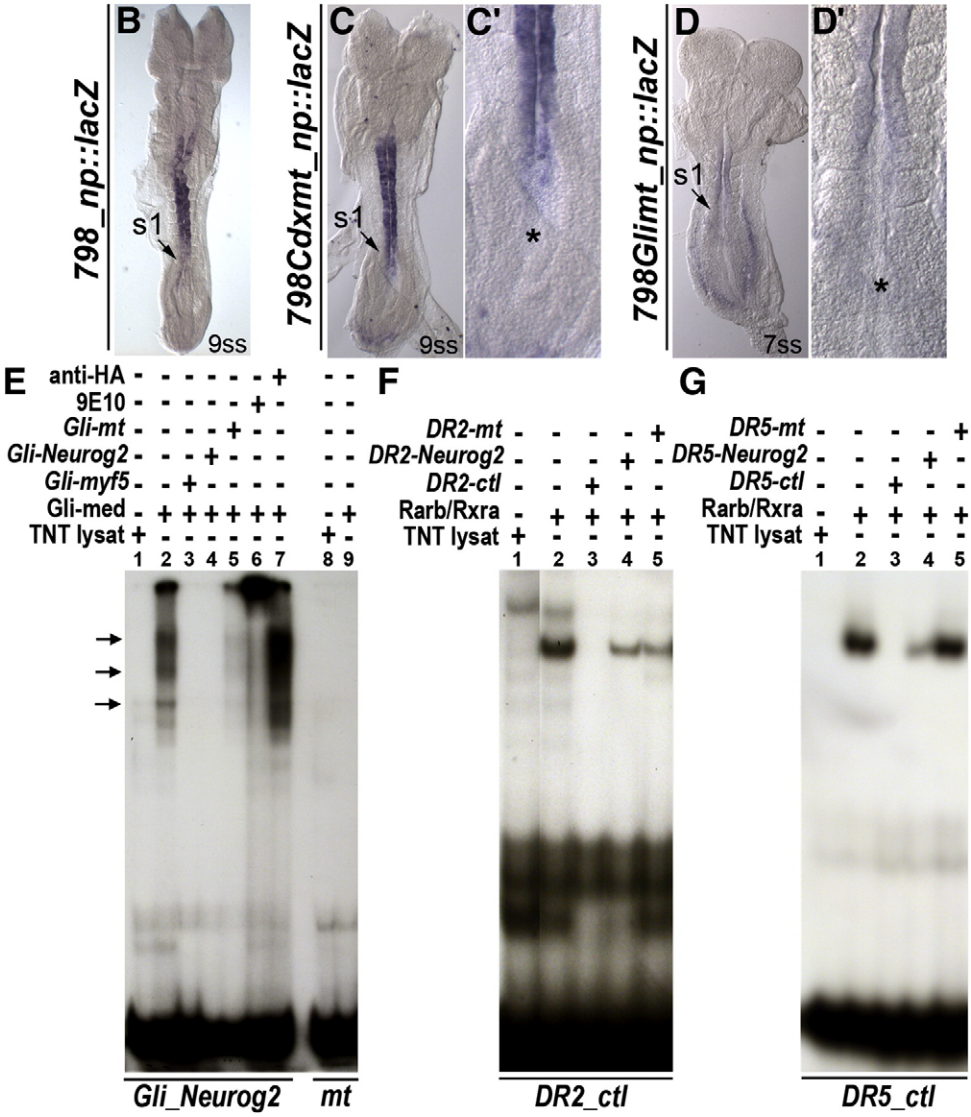

Fig. 5. Two RA response elements and one *Gli* binding site are necessary for the activity of the *Neurog-798* element. (A) Summary of the activity of the WT and mutated transgenes at different stages after ISH (E8.5) or X-gal staining (E9.5–E10.5). Insertions represent independent transgene insertions monitored in transient (T) or stable (S) transgenic embryos. (nd) not determined. (B–D′) WM ISH for *lacZ* showing that *Neurog2-798Cdxmt* enhancer activity (C, C′) recapitulates *Neurog-798* activity (B), whereas *Neurog2-798Glimt* is barely expressed within the *Neurog2* domain in the neural tube (D, D′). (E) EMSAs performed with WT or mutated *Gli* oligonucleotides (*Gli-Neurog2; Gli-mt*) incubated with a myc tagged Gli recombinant protein (Gli-med). The complexes (arrows) formed by the *Gli-Neurog2* oligonucleotides and the Gli proteins are specific. First, they can be displaced by an 100-fold molar excess of control *Gli-myf5* oligonucleotides (lane 3) or *Gli-Neurog2* oligonucleotides (lane 4). Second, the complexes are also displaced and accumulate in the well in the presence of the anti-myc antibody (9E10) (lane 6) but not in the presence of the anti-HA antibody (lane7). Third, *Gli-mt* is unable to form a complex with the Gli-med (lane 9). Of note, the addition of an 100-fold molar excess of *Gli-mt* oligonucleotides can although only partially, displace the *Gli-Neurog2*/Glimed complexes (lane 5). This suggests that the *Gli-mt* oligonucleotide retains a weak binding activity to the Gli-med. This differs from the results obtained by Gustafsson et al., 2002, probably because the sequences surrounding the core Gli binding site used in their study differs from the oligonuleotide used here. (F, G) EMSAs performed with control *Rare-DR2* or *DR5* oligonucleotides (*DR2-ctl, DR5-ctl*) incubated with heterodimers of recombinant RarbΔAB/RxrgΔAB proteins and an 100-fold molar excess of *DR2-ctl* (F, lane 3), *DR2-Neurog2* (F, lane 4), *DR2-Neurog2-mt* (F, lane 5), *DR5-ctl* (G, lane 3), *DR5-Neurog2* (G, lane 4), *DR5-Neurog2-mt* (G, lane5) oligonucleotides. *DR5-Neurog2* binds specifically RarbΔAB/RxrgΔAB heterodimers as an excess of *DR5-ctl or DR5-Neurog2* but not *DR5mt-Neurog2* competes for the *DR5-ctl* labelled oligonucleotides (G). This binding is of weak affinity since the labelled *DR5-Neurog2* oligonucleotides are unable to bind RarbΔAB/RxrgΔAB heterodimers. On the contrary, *DR2-Neurog2* does not specifically bind RarbΔAB/RxrgΔAB heterodimers, as an excess of either *DR2-Neurog2* or *DR2mt-Neurog2* is able to compete similarly the labelled *DR2-ctl* oligonucleotides (F). (asterisk) node; (s1) last formed somite.

modulators acting downstream of FGF signals. One possibility is that the modulation of *Neurog2* expression by FGF signalling involves cross-talks between FGF and other signalling pathways. A range of evidence suggest, for instance, that FGFs can inhibit quite rapidly the response of neural cells to Shh signalling in vitro (Fogarty et al., 2007) and ex vivo (Diez del Corral et al., 2003). In our experimental condition however, this mechanism is unlikely to account for the restriction of *Neurog2-798* activity and of *Neurog2* expression. Indeed, in mouse embryos the blockade of FGF signalling for 6 h does not increase significantly the activity of Shh signalling monitored by *Gli1* expression (10/13) (VR, ILR, PD; in preparation). Similarly, our results suggest that the effects of inhibiting FGF signalling on *Neurog2* expression do not result from a caudal expansion of RA activity. In agreement with these results, a recent study in mouse reported that the caudal boundary of RA activity monitored by the expression of a *Rare-lacZ* transgene is unchanged in mouse embryos depleted of FGF activity in the caudal structures from 6–7ss onwards (Wahl et al., 2007). Finally, the 'salt and pepper' pattern of activity of the *Neurog2-798* element could be reminiscent of a regulation by the Notch-mediated lateral inhibition process as it has been described at later stages (Bertrand et al., 2002). This input would allow the refinement of *Neurog2* early expression to a few scattered cells within the pNT and the newly formed neural tube and preserves a pool of progenitors in an uncommitted state. We identified at the 5′ end of the *Neurog2-798* element a conserved putative binding site (or Nbox) for the Notch downstream component Hes. In a pilot experiment we tested whether this binding site is involved in the regulation of *Neurog2-798* element activity 6 h after its electroporation in chick spinal cord. The activity of the *Neurog2-798* element deleted for the Nbox and of the WT element was similar ($n=6$ and $n=30$, respectively), strongly suggesting that the identified Nbox is not required for the early regulation of *Neurog2-798* element. Therefore, as for the FGF signalling, the sequences and transcriptional modulators underlying the regulation of *Neurog2* expression via *Neurog2-798* enhancer element by Delta-Notch signalling pathway still remain to be characterized.

FGF signalling maintains the undifferentiated state of a pool of self-renewing progenitors around the node during the elongation of the caudal structures and inhibits their migration within more anterior tissue. In this context, could the restriction of *Neurog2* expression via FGF signals contribute to the known functions of FGF in this area? Fate map analyses in chicken and mouse embryos show that neural and somitic progenitors overlap in the ectoderm lateral and caudal to the node (Brown and Storey, 2000; Cambray and Wilson, 2002, 2007; Psychoyos and Stern, 1996; Schoenwolf, 1992). In this region, FGF signalling via FGFR1 controls mesoderm cell fate and adhesive properties of the primitive streak cells (Ciruna and Rossant, 2001). As Neurog2 has been shown to regulate the migration properties of cortical neurons (Ge et al., 2006; Hand et al., 2005), it is tempting to hypothesise that *Neurog2* regulation mediated by FGFs could control the progression of precursor cells from the pNT to the neural tube. Videomicroscopy and cell tracing experiments on *Neurog2* mutant embryos would be required to confirm this hypothesis. In addition, in the spinal cord FGF signalling controls negatively the expression of ventral patterning genes and the process of neural differentiation via genes such as *Pax6* or *NeuroM* and *Neurog1,* respectively (Bertrand et al., 2000; Diez del Corral et al., 2002, 2003; Novitch et al., 2003; Olivera-Martinez and Storey, 2007). We show in this study that in the mouse embryo, *Neurog2* is the proneural gene that temporally controls the commitment and the process of differentiation of spinal progenitors from the 5ss onwards (Fig. 1). We propose that FGF signalling by negatively regulating *Neurog2* expression in the node region keeps the neural progenitors in an uncommitted state and prevent neurogenesis from occurring. This negative regulation could, therefore, permit the maintenance of progenitors in the domain of high FGF signalling.

The strict requirement of the *Rares* and *Gli* binding site for *Neurog2* induction raises the question of whether the integration of the activity of multiple transcription factors acting downstream of extracellular factors is a major mechanism underlying the genomic circuitry that defines the development of tissues. Surprisingly, only few studies have directly addressed this point in an in vivo context. In the fly, a unique combination of downstream components of signalling pathways together with transcription factors specify distinct cell fates (Flores et al., 2000; Halfon et al., 2000). In the mouse, the combination of Gli and Lef/Tcf binding sites regulates either in an antagonistic manner the expression of *Nkx2.2* in the motor neuron progenitor domain in the spinal cord (Lei et al., 2006) or in a synergistic manner the expression of *Myf5* in the muscle progenitors in the epaxial domain of the developing somites (Borello et al., 2006). Using a high-throughput method, Hallikas and coll. identified combinations of Gli and Lef/Tcf binding sites in conserved regulatory sequences of many genes suggesting that the integration of Shh and Wnt signalling pathways may be widely used to control gene expression during development (Hallikas et al., 2006). However, experimental data on specific enhancer elements are still needed to determine the function of these binding sites. A recent study described the regulation of *Cdx1* in paraxial mesoderm during early mouse embryogenesis by RA and Wnt signals. The authors further show the cooperative requirement of Rare and Lef/Tcf binding sites to mediate the full induction of *Cdx1* transcription (Pilon et al., 2007).

Our findings describe for the first time the cooperation between Rar/Rxr heterodimers and Gli proteins at the level of a single enhancer and we hypothesise that this specific cooperation could be widely used during neurogenesis for the generation of ventral neuronal subtypes. Remarkably, both RA and Shh signalling are required for the establishment of a large set of HD and bHLH genes in the ventral neural tube (Lupo et al., 2006). RA activity triggers the program of generic neural differentiation (Diez del Corral et al., 2003; Molotkova et al., 2005; Novitch et al., 2003; Wilson et al., 2004) and regulates directly or indirectly *Hox* gene expression (Bel-Vialar et al., 2002; Liu et al., 2001). Rar/Rxr heterodimers thus, are likely to be generic activators of transcription in distinct domains along the AP axis of the neural tube while the gradient of Gli activity along the DV axis throughout the neural tube (Stamataki et al., 2005) gives a spatial instructive information to ventral progenitors. Indeed, human and mouse embryonic stem cells require both RA and Shh signals to differentiate into motor neurons (Li et al., 2005; Wichterle et al., 2002). Furthermore, in the E8.5 embryos similarly to endogenous *Neurog2* expression, *Neurog2-798* activity localises to the ventral spinal cord (Fig. 4E). At a later stage (E10.5) *Neurog2-798* activity recapitulates only in part *Neurog2* endogenous expression and localises to the progenitor domains of the motor neurons and the p2 interneurons (Suppl. Fig. 3 and data not shown). In addition, mutations of the *Rares* or *Gli* binding sites abolish or severely reduce *Neurog2-798* activity at all stages observed (E8.5–E10.5, Fig. 5A, Suppl. Fig. 3), suggesting that these binding sites are also required for the activity of the enhancer in the motor neuron and p2 interneuron progenitor domains. The underlying mechanism by which Rar/Rxr heterodimers and Gli proteins regulate *Neurog2* transcription in the nucleus remains unknown. Interestingly, experiments performed on cell lines or from nuclear extracts showed that both transcription factors upon activation of the corresponding pathways interact with key transcriptional activators such as CBP and the Mediator complex and strongly promote transcription (Dilworth et al., 2000; Zhou et al., 2006). It is therefore tempting to hypothesise that these transcription factors cooperate in recruiting these chromatin modifiers and/or components of the basic transcription machinery to the *Neurog2-798* enhancer element and that this cooperation would be necessary to initiate *Neurog2* transcription (Pozzi et al., 2006).

In conclusion, our analysis has revealed that three regional signals cooperate at the level of a single enhancer and are responsible for the early expression of *Neurog2* in the posterior embryo. We also show that this gene plays a key role during the commitment of spinal progenitors. Hence, we anticipate that the combinatorial transcriptional activity of several signals is likely to be a general mechanism

underlying the temporally controlled generation of unique cell subtypes during vertebrate development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.003.

References

Akai, J., et al., 2005. FGF-dependent Notch signaling maintains the spinal cord stem zone. Genes Dev. 19, 2877–2887.
Akazawa, C., et al., 1992. Molecular characterization of a rat negative regulator with a basic helix–loop–helix structure predominantly expressed in the developing nervous system. J. Biol. Chem. 267, 21879–21885.
Bel-Vialar, S., et al., 2002. Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development 129, 5103–5115.
Bel-Vialar, S., et al., 2007. The on/off of Pax6 controls the tempo of neuronal differentiation in the developing spinal cord. Dev. Biol. 305, 659–673.
Bergsland, M., et al., 2006. The establishment of neuronal properties is controlled by Sox4 and Sox11. Genes Dev. 20, 3475–3486.
Bertrand, N., et al., 2002. Proneural genes and the specification of neural cell types. Nat Rev Neurosci. 3, 517–530.
Bertrand, N., et al., 2000. FGF signalling controls the timing of Pax6 activation in the neural tube. Development 127, 4837–4843.
Bettenhausen, B., et al., 1995. Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. Development 121, 2407–2418.
Bonnerot, C., et al., 1987. A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. Proc. Natl. Acad. Sci. U. S. A. 84, 6795–6799.
Borello, U., et al., 2006. The Wnt/beta-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. Development 133, 3723–3732.
Brown, J.M., Storey, K.G., 2000. A region of the vertebrate neural plate in which neighbouring cells can adopt neural or epidermal fates. Curr. Biol. 10, 869–872.
Bylund, M., et al., 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. Nat. Neurosci. 6, 1162–1168.
Cambray, N., Wilson, V., 2002. Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. Development 129, 4855–4866.
Cambray, N., Wilson, V., 2007. Two distinct sources for a population of maturing axial progenitors. Development 134, 2829–2840.
Charite, J., et al., 1998. Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. Development 125, 4349–4358.
Ciruna, B., Rossant, J., 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev. Cell. 1, 37–49.
Delfino-Machín, M., et al., 2005. Specification and maintenance of the spinal cord stem zone. Development 132, 4273–4283.
Diez del Corral, R., et al., 2002. Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development. 129, 1681–1691.
Diez del Corral, R., et al., 2003. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron. 40, 65–79.
Diez del Corral, R., Storey, K.G., 2004. Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. Bioessays. 26, 857–869.
Dilworth, F.J., et al., 2000. ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro. Mol. Cell. 6, 1049–1058.
Flores, G.V., et al., 2000. Combinatorial signaling in the specification of unique cell fates. Cell. 103, 75–85.
Fode, C., et al., 2000. A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. Genes Dev. 14, 67–80.
Fogarty, M.P., et al., 2007. Fibroblast growth factor blocks Sonic hedgehog signaling in neuronal precursors and tumor cells. Proc. Natl. Acad. Sci. U. S. A. 104, 2973–2978.
Ge, W., et al., 2006. Coupling of cell migration with neurogenesis by proneural bHLH factors. Proc. Natl. Acad. Sci. U. S. A. 103, 1319–1324.
Germain, P., et al., 2002. Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. Nature 415, 187–192.
Goodrich, L.V., et al., 1999. Overexpression of ptc1 inhibits induction of Shh target genes and prevents normal patterning in the neural tube. Dev. Biol. 211, 323–334.
Gradwohl, G., et al., 1996. Restricted expression of a novel murine atonal-related bHLH protein in undifferentiated neural precursors. Dev. Biol. 180, 227–241.
Guillemot, F., 2007. Spatial and temporal specification of neural fates by transcription factor codes. Development 134, 3771–3780.
Gustafsson, M.K., et al., 2002. Myf5 is a direct target of long-range Shh signaling and Gli regulation for muscle specification. Genes Dev. 16, 114–126.
Halfon, M.S., et al., 2000. Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. Cell 103, 63–74.
Hallikas, O., et al., 2006. Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. Cell 124, 47–59.
Hand, R., et al., 2005. Phosphorylation of Neurogenin2 specifies the migration properties and the dendritic morphology of pyramidal neurons in the neocortex. Neuron 48, 45–62.
Henrique, D., et al., 1997. cash4, a novel achaete-scute homolog induced by Hensen's node during generation of the posterior nervous system. Genes Dev. 11, 603–615.
Incardona, J.P., et al., 1998. The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. Development 125, 3553–3562.
Lardenois, A., et al., 2006. PromAn: an integrated knowledge-based web server dedicated to promoter analysis. Nucleic Acids Res. 34, W578–W583.
Lee, S.K., et al., 2005. Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. Genes Dev. 19, 282–294.
Lei, Q., et al., 2006. Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. Dev. Cell. 11, 325–337.
Li, X.J., et al., 2005. Specification of motoneurons from human embryonic stem cells. Nat. Biotechnol. 23, 215–221.
Liu, J.P., et al., 2001. Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron. 32, 997–1012.
Lohnes, D., 2003. The Cdx1 homeodomain protein: an integrator of posterior signaling in the mouse. Bioessays 25, 971–980.
Louvi, A., Artavanis-Tsakonas, S., 2006. Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. 7, 93–102.
Lu, Q.R., et al., 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 109, 75–86.
Lupo, G., et al., 2006. Mechanisms of ventral patterning in the vertebrate nervous system. Nat. Rev. Neurosci. 7, 103–114.
Ma, Y.-C., et al., 2008. Regulation of motor neurons specification by phosphorylation og Neurogenin 2. Neuron 58, 65–77.
Marshall, H., et al., 1994. A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature 370, 567–571.
Mathis, L., et al., 2001. FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. Nat. Cell. Biol. 3, 559–566.
Mathis, L., Nicolas, J.F., 2000. Different clonal dispersion in the rostral and caudal mouse central nervous system. Development 127, 1277–1290.
Minowada, G., et al., 1999. Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 126, 4465–4475.
Mizuguchi, R., et al., 2001. Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron 31, 757–771.
Mohammadi, M., et al., 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276, 955–960.
Molotkova, N., et al., 2005. Requirement of mesodermal retinoic acid generated by Raldh2 for posterior neural transformation. Mech. Dev. 122, 145–155.
Novitch, B.G., et al., 2001. Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron 31, 773–789.
Novitch, B.G., et al., 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. Neuron 40, 81–95.
Olivera-Martinez, I., Storey, K.G., 2007. Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension. Development 134, 2125–2135.
Pilon, N., et al., 2007. Wnt signaling is a key mediator of Cdx1 expression in vivo. Development 134, 2315–2323.
Pozzi, S., et al., 2006. RAR-mediated epigenetic control of the cytochrome P450 Cyp26a1 in embryocarcinoma cells. Oncogene 25, 1400–1407.
Psychoyos, D., Stern, C.D., 1996. Fates and migratory routes of primitive streak cells in the chick embryo. Development 122, 1523–1534.
Rossant, J., et al., 1991. Expression of a retinoic acid response element-hsplacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev. 5, 1333–1344.
Roztocil, T., et al., 1997. NeuroM, a neural helix–loop–helix transcription factor, defines a new transition stage in neurogenesis. Development 124, 3263–3272.
Sandberg, M., et al., 2005. Sox21 promotes the progression of vertebrate neurogenesis. Nat. Neurosci. 8, 995–1001.
Scardigli, R., et al., 2003. Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. Development 130, 3269–3281.
Scardigli, R., et al., 2001. Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. Neuron. 31, 203–217.
Schoenwolf, G.C., 1992. Morphological and mapping studies of the paranodal and postnodal levels of the neural plate during chick neurulation. Anat. Rec. 233, 281–290.
Simmons, A.D., et al., 2001. Neurogenin2 expression in ventral and dorsal spinal neural tube progenitor cells is regulated by distinct enhancers. Dev. Biol. 229, 327–339.

Soustelle, L., et al., 2007. Neurogenic role of Gcm transcription factors is conserved in chicken spinal cord. Development 134, 625–634.
Stamataki, D., et al., 2005. A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. Genes Dev. 19, 626–641.
Stern, C.D., et al., 2006. Head-tail patterning of the vertebrate embryo: one, two or many unresolved problems? Int. J. Dev. Biol. 50, 3–15.
Vermot, J., et al., 2005. Retinaldehyde dehydrogenase 2 and Hoxc8 are required in the murine brachial spinal cord for the specification of Lim1+motoneurons and the correct distribution of Islet1+motoneurons. Development 132, 1611–1621.
Yee, S.P., Rigby, P.W., 1993. The regulation of myogenin gene expression during the embryonic development of the mouse. Genes Dev. 7, 1277–1289.
Wahl, M.B., et al., 2007. FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. Development 134, 4033–4041.
Wichterle, H., et al., 2002. Directed differentiation of embryonic stem cells into motor neurons. Cell 110, 385–397.
Wilson, L., et al., 2004. Retinoic acid and the control of dorsoventral patterning in the avian spinal cord. Dev. Biol. 269, 433–446.
Zhou, H., et al., 2006. Mediator modulates Gli3-dependent Sonic hedgehog signaling. Mol. Cell. Biol. 26, 8667–8682.
Zhou, Q., Anderson, D.J., 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. Cell 109, 61–73.